Anti-HCMV and KSHV effect of a trapping ligand antagonist for Herpesvirus-encoded GPCR

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We have found and synthesized a trapping ligand peptide H22-LP (the conservative sequence is NAHCALL) from a random phage library according to the broad-spectrum trapping receptor H22, which derived from the residue 14-35 near the N-terminal region of receptor US28 on HCMV. In this study, we will evaluate its potential as an efficient antagonist of US28 and the anti-virus activity, acting as a broad spectrum chemokine receptors antagonist. Stable expression of US28 and ORF74 in NIH/3T3 cells were successfully constructed in vitro. Flow cytometry was used to determine the concentration of Ca\(^{2+}\) induced by H22-LP, and the binding of H22-LP and US28 was confirmed by enzyme-linked immunosorbent assay (ELISA). Antivirus activity of H22-LP on HCMV and KSHV was evaluated by anti-virus experiments. Our data suggest that H22-LP is an effectual antagonist of receptor US28 of HCMV and ORF74 of KSHV in the transfection assay, and it has potential to inhibit infection of HCMV and KSHV. These results provide support for the development of anti-virus strategies based on targeted inhibiting the infection of herpesvirus.

Keywords: Antagonist, Human cytomegalovirus, Kaposi’s sarcoma-associated herpesvirus ORF74, Peptide H22-LP, Trapping receptor/ligand, US28

Human cytomegalovirus (HCMV), a member of the β-herpesvirus family, is widely present among the general population and the rate of infection is up to 90%\(^1\). The HCMV infection can cause severe pathologies in immune-compromised patients (e.g. HIV-infected patients, organ transplant recipients, newborn infants), while being asymptomatic in immune-competent individuals\(^2,3\). Although HCMV infection of healthy children and adults is usually asymptomatic, it is a leading cause of birth defects and a major cause of morbidity and mortality in immune-compromised individuals. HCMV has developed a variety of strategies to evade the immune system, which refers to the communication between numerous virus genes and cells. The HCMV genome, the largest of any human virus (236 kb), known to date is divided into two, a long part and a short part named UL and USrespectively. Virus genes encode potential G-protein-coupled receptors (GPCRs) or GPCR ligands, including US27, US28, UL33, and UL78. In contrast to UL33 and UL78 genes, which have counterparts in all sequenced β-herpesvirus including HHV-6 and HHV-7, the US27 and US28 are matchless for primate CMVs closely related to HCMV\(^4\). Only US28 has been shown to bind to chemokines, and has been most intensively studied. US28 shows the highest homology (33%) to the CC chemokine, including CCL5 and CCL2. Moreover, it can also bind to CX3C family, especially CX3CL1\(^5\). It’s obvious that US28 belongs to a broad spectrum receptor. US28 can regulate intercellular transfer of HCMV via binding membrane-associated CXCL1 (fractalkine). Additionally, US28-induced vascular smooth muscle cell (SMC) migration may play a key role in induction of vascular diseases, which could provide the molecular basis for the implication of HCMV in atherosclerosis. Therefore, US28 nonetheless plays an important role in the development of anti-HCMV therapy.

Kaposi’s sarcoma-associated herpesvirus (KSHV, human herpesvirus8), a member of γ-herpesvirus family, was first identified by DNA analysis of Kaposi’s sarcoma (KS) in biopsy tissues from AIDS patients\(^6\). Three different variants of KS, referring to the classic KS, endemic KS and AIDS-associated KS, are recognized. Classic KS doesn’t spread beyond the extremities and endemic KS is common in particular parts of Africa, while AIDS-associated KS is a particularly aggressive and often fatal variant of KS.
Moreover, KSHV is linked to primary effusion lymphoma (PEL) and multicentric Castleman’s disease (MCD). The infection cycle is divided into two phases, viz., latent and lytic phases. KSHV locates in the latent phase in KS and PEL, as well as lytic replication is prominent in MCD.

The KSHV genome encodes 84 open-reading frames and 12 microRNAs (miRNAs). The G-protein-coupled receptor (GPCR) specified by open reading frame 74 (ORF74) of human herpesvirus8 (HHV-8) is a close homologue of CXCR2 and binds to chemokines of CXC family, which is indicative for a role in the avoidance of the host immune system by KSHV. The HHV-8 GPCR is able to activate several lytic cycle promoter in transfection assays. ORF74 acts as a potential mediator of HHV-8 associated diseases such as KS, PEL and MCD.

Antiviral strategies directed at targeted inhibiting or killing of herpesvirus based on the expression of virus G-protein-coupled receptor (vGPCR) have been studied. The homology of US28 to other vGPCRs and representative chemokines of herpesvirus is 20—95% in BLAST and HHV-8 ORF74 shows the highest homology (95%). In a previous study, a peptide, based on the broad-spectrum chemokine binding activity of US28 was designed, from the N-terminal active site and the transmembrane domain of US28 by bioinformatics methods. The sequence of residue 14-35 near the N-terminal region of US28 was Phe-Asp-Tyr-Asp-Ala-Th-Pro-Cys-Val-Phe-Thr-Asp-Val-Leu-Asn-Gln-Ser-Lys-Pro-Val, which named H22. H22, called trapping receptor, acted as an analoguous site of vGPCR of herpesvirus. It exhibited no chemotactic effect and could block the binding between the physiologically active chemokine and receptor. At the same time, using a random phage library including 25 chemokines, 30 positive clones were selected. The competitive blocking and competitive inhibition showed nine positive clones, which blocked by hMIP-1β when combined with synthetic peptide H22. DNA sequence analysis of the nine positive clones exhibited similar conservative sequences (NAHCALL), which named H22-LP. In contrast to trapping receptor H22, we describe H22-LP as trapping ligand, synthesized by Guangzhou Huatuo Bio-tech Development Co, Ltd. In the present report, the broad spectrum antivirus function of H22-LP has been described by further evaluating its potential as an efficient antagonist of US28 and ORF74, for possible use in treatment of diseases caused by herpesvirus.

Materials and Methods

Cells and virus—HELF cells (human embryonic lung fibroblast) were cultured in DMEM with 10% fetal bovine serum (FBS), 200 mM/L glutamine and mycillin supplement at 37 °C, 5% CO₂ atmosphere. NIH/3T3 cells were also incubated in DMEM with 10% fetal bovine serum (FBS) in a humidified 5% CO₂ incubator at 37 °C.

Two hundred nintythree cells (derived from human embryonic kidney) were cultured in DMEM supplement with 10% FBS, 2 mM L-glutamine and amino acid.

BCBL cells (conserved in the laboratory) harboring KSHV were maintained in RPMI 1640 medium supplement with 10% FBS, 2 mM L-glutamine, 100 U penicillin, and 10U streptomycin.

HCMV (human cytomegalovirus) AD169 virus solution 5-10 µl was added to HELF cells in logarithmic phase with a concentration of 1×10⁹/mL. Cytotoxicity of cells was observed daily at inverted microscope, and pp65 was monitored every 3 days. After 8 days, the virus antigen were tested, then digested, centrifuged, and cryopreserved at -80 °C. TCID₅₀ was 10⁻⁴.⁶⁷/100µl for HCMV AD169.

Cell lines US28-NIH/3T3 and ORF74-NIH/3T3 construction—The PCR reaction was obtained from AD169 HCMV liquid, which was boiled for 10 min and centrifuged at 12,000 rpm for 5 min. The DNA sequence encoding US28 was isolated by PCR with primers specific for US28 5'-GCCAACGCTTATGACACCCGACAGCA-3' and 5'-TGCTCTAGATTACCGTATAATTTTGTAGACG-3' (restriction sites are underlined and italicized). The products were digested by Hind III and Xba I and ligated into the Xba I/Hind III sites of the expression vector plasmid pcDNA3.1 (bought from ATCC, USA). Stable US28-NIH/3T3 cell lines, which transfer the US28 gene into NIH/3T3 cells, were constructed by calcium phosphate. Three days later, 400 µg/mL G-418 disulfide was added to the culture medium when the cells began to die in the control group. Recombinant clones were screened and cryopreserved at -80 °C.
The DNA encoding for ORF74 was extracted from cell line BCBL infected with HHV-8 and amplified by PCR with the primers for ORF74 which contained restriction sites for Bgl (5'-CGATGAGATCTGGCTACGTGGTGCG-3') and EcoRI (5'-CGCATGAATTCCCTTATTGTTAGGCGATGGC-3'). ORF74 gene was ligated into Bgl/EcoRI sites of plasmid pcEFL (bought from ATCC, USA) and transfected into NIH/3T3 cells.

Flow cytometry analysis—The transfected cells were cultured in fresh medium at 37 °C, 5% CO₂ atmosphere in advance. Aliquots (2.0x10⁶) of transfected cells were washed twice with serum-free medium. Cells treated with hMIP-1β (10 ng/mL) were included as a positive control for induction of increased calcium concentration. Cells deal with H22-LP (100 ng/mL) were referred as the inhibitor group, while both H22-LP (100 ng/mL) and hMIP-1β (10 ng/mL) were named as the inhibitor plus chemokine group, which was treated with H22-LP for 30 min at 4 °C in advance. After the cells were incubated for 24 h, they (1.0x10⁷ cells/ml) were washed with serum-free medium and labeled with Fluo-3/AM (10 μmol/L). Subsequently, cells were cultured in dark for 30 min and washed twice with phosphate buffered saline (PBS). Samples were analyzed using the FACSCalibur flow cytometer.

Cross-linking assay—The combination between H22-LP and receptors US28 and ORF74 was determined by ELISA. H22-LP was diluted (100 ng/ml) and added to the 96-well microtiter plate (100 μl/well). BSA was used as the positive group and 10 ng/ml hMIP-1β acted as the negative group. After incubating overnight at 4 °C, the unbound H22-LP were washed with PBST, then blocked with PBST-2% BSA (200 μl/well) at 37 °C for 1 h and washed again. The transfected cells (US28-NIH/3T3 or ORF74-NIH/3T3 cell lines) were adjusted to 1.0x10⁷ cells/ml and incubated for 1 h at 37 °C with pp65 monoclonal antibody (bought from Chemicon, USA) in 96-well microtiter plate (100 μl/well), which was diluted with PBST containing BSA. Cells were washed thrice with PBST and incubated for 1h at room temperature with goat anti-mouse HRP-IgG secondary antibody (bought from Novagen, Germany), which diluted with BSA-PBST. Subsequently, cells were washed three times again and incubated with TMB (100 μl/well) for 10 min at room temperature. The reaction was stopped with 1 M H₂SO₄, and absorption at 450 nm was determined. All readings were done in duplicate.

Cytotoxicity assay of H22-LP—Cell toxicity was quantified using a MTT assay. Briefly, HELF cells or 293 cells were seeded into 96-well plates at a density of 5x10⁴ cells/ml, incubated at 37 °C in a 5% CO₂ atmosphere for 24 h, until 90% or greater confluency of the monolayers was reached. Increasing concentrations of H22-LP (from 0.01 to 1000 μg/ml) were added to the cells, with a replicate number of three wells per concentration. After 24 h, the liquid were removed and cells were incubated with MTT for 4 h. Subsequently, the blue formazan product was removed by adding 100 μl/well DMSO. The absorbance at 490 nm was determined using a microplate reader, and the cell viability was calculated.

Inhibition of infectious virus production assay—To determine inhibitory effect of H22-LP on HCMV, increasing concentrations of H22-LP (100 μl/well), 80 μl equivalent number of HELF cells and the supernants of HCMV (20 μl) were added into the 96-well plates. The total volume was 200 μl/well. The plates were incubated at 37 °C in a 5% CO₂ humidified atmosphere and observed daily for cytopathic effect (CPE) using the microscope. After five days, the CPE was observed in all virus control group, the number of CPE in each well was counted for every concentration. The percentage of wells with CPE was calculated and the EC₅₀ (50% effective concentration) was determined using Reed-Muench.

To determine inhibitory effect of H22-LP on KSHV, 293 cells (2x10⁵ cells/well) were seeded in 24 wells plates and infected with the supernants containing KSHV, which was released from BCBL cells using 0.6 mM valproate. At the same time, different concentrations of peptide H22-LP (200μl/well) were added (500 μl total volume) and incubated at 37 °C for 3 h, then the liquid was removed following 500 μl fresh medium. Subsequently, the plates were incubated at 37 °C in a 5% CO₂ atmosphere for 48 h. The number of infected target cells was determined by flow cytometry and infectivity is expressed as percentage of target cells that scored GFP-positive.

Results

Synthesis of H22-LP—H22-LP had been synthesized by the Guangzhou Huatuo Bio-tech Development Co, Ltd, China. The purity of H22-LP,
determined by high-performance liquid chromatography (HPLC), was more than 98% (Fig. 1A). The molecular weight was calculated by mass spectrometry as 977.5 (Fig. 1B), in accordance with the theoretical value.

The expression of cell lines US28-NIH/3T3 and ORF74-NIH/3T3—The stable cell lines US28-NIH/3T3 and ORF74-NIH/3T3 have been successfully constructed. The PCR products of US28, digested with Hind III and Xba I, were shown as Fig. 2A. Cell line US28-NIH/3T3 was expressed and analyzed by Western blot (Fig. 2B). The PCR products of ORF74, digested with Bgl and EcoRI, and Western blot of ORF74-NIH/3T3 are shown as Fig. 3A and B respectively.

Prevention the evaluation of Ca\(^{2+}\) induced by hMIP-1β—Flow cytometry was used to test the ability of H22-LP to bind to receptor US28, while blocked

Fig. 1—Determination of the purity and molecular weight of H22-LP. (A: The representative HPLC spectrum of H22-LP; B: MS spectrum of H22-LP)
the binding between US28 and hMIP-1β. hMIP-1β (10 ng/mL) can significantly increase the concentration of Ca²⁺ ($P<0.01$; Table 1). Pretreatment with 100 ng/ml H22-LP can prevent the increase of Ca²⁺ induced by hMIP-1β ($P<0.05$).

**Binding H22-LP with US28 and ORF74**—A450 value of H22-LP treatment (approximately 0.6) was much higher than the BSA group ($P<0.01$; Table 2), which is below 0.15. The result demonstrated that a specific molecular interaction between H22-LP and receptors (US28 and ORF74).

**Inhibition of infectious virus production by H22-LP**—In order to assess the cytotoxic effects of H22-LP, MTT assay was performed after incubating HELF or 293 cell cultures with increasing concentrations. No significant cytotoxicity effects were observed even if the highest concentration and the survival rate of each treatment group was over 80% (Fig. 4).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Fluorescence</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>805±47.21</td>
</tr>
<tr>
<td>hMIP-1β (10 ng/ml)</td>
<td>1509±69.15</td>
</tr>
<tr>
<td>H22-LP (100 ng/ml)</td>
<td>1104±37.20</td>
</tr>
<tr>
<td>H22-LP (100 ng/ml)+hMIP-1β (10 ng/ml)</td>
<td>927±78.17</td>
</tr>
</tbody>
</table>

Compared with control group, $^aP<0.01$; Compared with positive group, $^bP<0.05$

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (ng/ml)</th>
<th>US28</th>
<th>ORF74</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMIP-1β</td>
<td>10</td>
<td>0.603±0.091*</td>
<td>0.598±0.068*</td>
</tr>
<tr>
<td>H22-LP</td>
<td>100</td>
<td>0.581±0.086*</td>
<td>0.579±0.039*</td>
</tr>
<tr>
<td>BSA</td>
<td>Control</td>
<td>0.120±0.019</td>
<td>0.131±0.012</td>
</tr>
</tbody>
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*Compared with control, $P<0.01$
The anti-HCMV activity was evaluated by CPE in presence of various concentrations of H22-LP. The 50% effective concentration (EC\textsubscript{50}) of H22-LP was 0.53 ng/ml, greater than the GCV group (0.68 ng/mL) (Fig. 5).

The effect of H22-LP on release of infectious virus from BCBL cells, which carries KSHV genome was examined (Fig. 6). Virion release was stimulated by treatment for 4 days with 0.6 mM valproate. The amounts of virus were quantitated by incubating with susceptible 293 target cells. The number of GFP-positive cells after 2 days was scored. Fig.6 shows that H22-LP produced dose-dependent inhibition, with much greater than the control group. The EC\textsubscript{50} value of H22-LP on KSHV was 3.41 ng/mL.

Discussion
The chemokine system is highly redundant, with many chemokines hitting one receptor. At the same time, receptors responding to the same chemokine. A great of chemokine receptor antagonists have been applied to animal experiments or clinical studies in recent years\textsuperscript{13}. Screening of small molecules chemokine receptors antagonists, which bind to virus-encoded chemokine receptors but do not initiate cytoplasmic signal transduction cascades, is an important consideration for broad spectrum anti-virus therapies. The N-terminal region of a chemokine, initiating a series of signal cascades by binding to receptors, can be referred as a candidate site of antagonists. According to the above theory, a small molecule peptide H22-LP was designed, which derived from the US28 N-terminal region of HCMV.

The designation of peptide H22-LP was, first, based on the US28 receptor, which shows high homologous to multiple herpes virus family members such as ORF74 of HHV-8, U12 of HHV-7. Second, US28 is a broad spectrum receptor, which can bind to a variety of human chemokines, such as the CC and CX3C chemokine. The initial purpose of the present study on the screening was to develop a small peptide acting as a broad spectrum chemokine antagonist, which combines to various chemokine receptors. In the present study, its potential as an efficient antagonist of US28 and ORF74, and its anti-herpes virus activity have been evaluated.

The results revealed that (i) the inhibitor group (H22-LP) up-regulated the Ca\textsuperscript{2+} concentration, nevertheless, pretreatment with H22-LP could obviously inhibit the increasing of Ca\textsuperscript{2+} concentration induced by hMIP-1\textbeta. These finding demonstrate a fact that H22-LP could bind to the US28 receptor on NIH/3T3 cells instead of hMIP-1\textbeta, with an emphasis on H22-LP was an effective antagonist of US28. This agrees with previous findings that the CC chemokines could increase the intracellular concentration of Ca\textsuperscript{2+}\textsuperscript{14}. (ii) In contrast to the BSA group, the H22-LP group produced noticeable differences of absorbance, suggesting a special molecular interaction existed between peptide H22-LP and receptors (US28 and ORF74). (iii) H22-LP was first examined for its inhibitory effects on HCMV, and its anti-HCMV activity was demonstrated through CPE. The effective concentrations of H22-LP was investigated and found
that the EC$_{50}$ for HCMV production was 0.53 ng/ml, whereas the EC$_{50}$ for GCV was 1.86 µg/ml. At the same time, anti-KSHV activity was examined through scoring the number of GFP-positive cells by flow cytometry. There is no plaque assay available for KSHV and the virally infected cell culture does not exhibit great CPE$^{15}$, therefore, this assay may be exclusively for KSHV. The result showed that H22-LP produced dose-dependent inhibition of infectious virus release and the EC$_{50}$ was 3.41 ng/ml.

The HCMV genome encodes four vGPCRs: US27, US28, UL33 and UL78, all of these genes encode chemokine receptor homologues. Moreover, high homologues and structure similarity of US28 exist among U12, U51, and ORF74 of HHV-6, HHV-7 and HHV-8 (KSHV) respectively$^{16}$. It can be concluded that H22-LP may be a small molecule broad spectrum antivirus peptide. In this article, the US28-NIH/3T3 and ORF74-NIH/3T3 cell lines were constructed successfully in vitro. Flow cytometry was used to detect the wave of Ca$^{2+}$ concentration induced by H22-LP, and the binding of H22-LP and US28 was confirmed by cross-linking assay. In addition to this, H22-LP showed antivirus effect, which only referred to HCMV and KSHV. Antivirus activity of H22-LP on other herpes virus family will be further determined.

Overall, these results suggest that H22-LP is an efficient antagonist of receptor US28 of HCMV, and it has potential to inhibit the infection of HCMV and KSHV. The strategy of targeting to inhibit or kill herpesvirus to treat disease will continue to grow as increasing numbers of small molecule peptide of chemokine receptors antagonist are developed. H22-LP might be useful as potential therapeutic agent against herpesvirus infection.

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References